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STUDIES ON FACTORS MODULATING GLUCOSE HOMEOSTASIS IN HEALTHY AND DIABETIC RATS

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To my family

ABSTRACT

Glucose is the most common substrate for energy metabolism. Despite the varying demands for glucose, the body needs to regulate its internal environment and maintain a constant and stable condition. Glucose homeostasis requires harmonized interaction between several tissues, achieving equilibrium between glucose output and uptake. In this thesis we aimed to investigate factors modulating glucose homeostasis in a rat model of type 2 diabetes, the Goto-Kakizaki (GK) rat. In addition, we investigated sex differences in hepatic carbohydrate and lipid metabolism in healthy rats.

In Paper I, three-week but not three-day treatment with a Southeast Asian herb, *Gynostemma pentaphyllum* (GP), significantly reduced plasma glucose (PG) levels in GK rats. An intra-peritoneal glucose tolerance test (IPGTT) was significantly improved in GP-treated compared to placebo-treated group. In the GP treated rats, the glucose response in an intra-peritoneal pyruvate tolerance test was significantly lower, indicating decreased gluconeogenesis, and hepatic glucose output (HGO) was reduced. GP-treatment significantly reduced hepatic glycogen content, but not glycogen synthase activity. The study provides evidence that the GP extract exerted anti-diabetic effect in GK rats, reducing PG levels and HGO, suggesting that GP improves the hepatic insulin sensitivity by suppressing gluconeogenesis.

In Paper II, shikonin, a naphthoquinone derived from the Chinese plant *Lithospermum erythrorhizon*, increased glucose uptake in L6 myotubes, but did not phosphorylate Akt. Furthermore we found no evidence for the involvement of AMP activated protein kinase (AMPK) in shikonin induced glucose uptake. Shikonin increased the intracellular levels of calcium in these cells and stimulated the translocation of GLUT4 from intracellular vesicles to the cell surface in L6 myotubes. In GK rats treated with shikonin once daily for 4 days, PG levels were significantly decreased. In an insulin sensitivity test, the absolute PG levels were significantly lower in the shikonin-treated rats. These findings suggest that shikonin increases glucose uptake in muscle cells via an insulin-independent pathway dependent on calcium.

In Paper III, GK and control Wistar rats were injected daily for up to 4 weeks with either a non-hematopoietic erythropoietin analog ARA290 or with placebo. PG levels in GK but not Wistar rats were significantly lower in ARA290-treated compared to placebo. After 2 and 4 weeks, the IPGTT was significantly improved in ARA290 treated GK rats. In insulin and pyruvate tolerance tests, glucose responses were similar in ARA290 and placebo groups. In isolated GK rat islets, glucose-stimulated insulin release was two-fold higher and islet intracellular calcium concentrations in response to several secretagogues were significantly higher in ARA290-treated than in placebo-treated GK rats. These findings indicate that treatment with ARA290 significantly improved glucose tolerance in diabetic GK rats, most likely due to improvement of insulin release.

In Paper IV, sex differences in hepatic carbohydrate and lipid metabolism were characterized in healthy rats. No sex-differences were observed regarding hepatic triglyceride content, fatty acid oxidation rates or insulin sensitivity. Male rats had higher ratios of insulin to glucagon levels, increased hepatic glycogen content, a lower degree of AMPK phosphorylation, a higher rate of glucose production and higher expression levels of gluconeogenic genes, as compared to female rats. A sex-dependent response to mild starvation was observed with males being more sensitive. In conclusion, sex-differences reflect a higher capacity of the healthy male rat liver to respond to increased energy demands.

Key words: glucose homeostasis, type 2 diabetes, GK rats, L6 myotubes, hepatic glucose output, insulin sensitivity, sex differences.

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LIST OF PUBLICATIONS

I. **Yassin K,** Huyen V, Hoa NK, Östenson CG: Herbal extract of Gynostemma pentaphyllum decreases hepatic glucose output in type 2 diabetic Goto-Kakizaki rats. Int J Biomed Sci 2011; 7 (2): 131-136.

II. Oberg AI*, **Yassin K*,** Csikasz RI, Dehvari N, Shabalina IG, Hutchinson DS, Wilcke M, Ostenson CG, Bengtsson T: Shikonin increases glucose uptake in skeletal muscle cells and improves plasma glucose levels in diabetic Goto-Kakizaki rats. PLoS ONE 2011;6:e22510.

III. **Yassin K,** Li LS, Palmblad M, Efendic S, Berggren PO, Cerami A, Östenson CG: The non-hematopoietic erythropoietin analogue, ARA290, improves glucose tolerance and insulin release in GK rats, a model of type 2 diabetes Manuscript.

IV. Gustavsson C, **Yassin K,** Wahlstrom E, Cheung L, Lindberg J, Brismar K, Ostenson CG, Norstedt G, Tollet-Egnell P: Sex-different hepatic glycogen content and glucose output in rats. BMC Biochemistry 2010;11:38.

*Authors contributed equally to the work.

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LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AUCs	Areas under the curves
DMEM	Dulbecco's modified Eagle's medium
DPP-IV	dipeptidyl peptidase-IV
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
GLUTs	Glucose transporters
GP	*Gynostemma pentaphyllum*
HbA1c	Glycosylated haemoglobin
HGO	Hepatic glucose output
HOMA-IR	Homeostasis Model of Assessment-Insulin Resistance
i.p.	Intraperitoneally
IPGTT	Intraperitoneal glucose tolerance test
IPPTT	Intraperitoneal pyruvate tolerance test
IR	Insulin resistance
KRB	Krebs-Ringer bicarbonate buffer solution
LE	*Lithospermum erythrorhizon*
PBS	Phosphate-buffered saline
PG	Plasma glucose
PI3K	Phosphatidylinositol 3-kinase
PTP1B	Phosphotyrosine phosphatase 1B
RT-PCR	Real –time polymerase chain reaction PCR
s.c.	Subcutaneously
SCITT	Subcutaneous insulin tolerance test
SD	Sprague-Dawley rats
SUs	Sulfonylureas
T2D	Type 2 diabetes
TG	Triglyceride
W	Wistar

Abbreviations used only once are described where they appear in the text.

1 BACKGROUND

1.1 GLUCOSE HOMEOSTASIS

1.1.1 Physiology of plasma glucose concentration

Glucose is the most common substrate for energy metabolism. Certainly, under normal circumstances glucose is the only form of energy that can be used by the brain and central nervous system (Thorens and Mueckler, 2010, Pellerin and Magistretti, 2003). The amount of glucose in the circulation is dependent upon a balance of the rate of glucose entering the circulation (glucose appearance) and the rate of glucose removal from the circulation (glucose disappearance) (Gerich, 2000). Circulating plasma glucose is derived from intestinal absorption, glycogenolysis, the breakdown of glycogen, which is the storage form of glucose, and gluconeogenesis, the formation of glucose primarily from lactate, glycerol and amino acids during the fasting state (Saltiel and Kahn, 2001). Despite the varying levels of glucose after a meal or during fasting and exercise, the body needs to regulate its internal environment and maintain a constant and stable condition (Vidal-Puig and O'Rahilly, 2001). Hence, blood glucose levels are under the regulation of various homeostatic control mechanisms which maintain them within a narrow range in both the fasting and feeding states.

The glucoregulatory hormones in glucose homeostasis require a harmonized interaction between several tissues, achieving equilibrium between glucose output and uptake. Insulin is the major regulator of glucose metabolism, although its actions are modified in many respects by counter-regulatory hormones (glucagon, adrenaline, cortisol and growth hormone).

To maintain circulating glucose concentrations in a relatively narrow range, insulin decreases blood glucose levels by suppressing hepatic glucose output and facilitating glucose uptake in liver and extrahepatic tissues (muscle and adipose tissue) (Thorens and Mueckler, 2010, Pessin and Saltiel, 2000). This regulation depends on the state of the body and can be modified by counter-regulatory hormones, which increase blood glucose concentrations. The effect of counter-regulatory hormones is to cause greater production of glucose from the liver and less utilization of glucose in adipose and muscle tissues. During fasting, the liver produces glucose both by glycogenolysis and gluconeogenesis, whereas in the fed state the liver takes up glucose and stores glucose as glycogen. Skeletal muscle, due to its large mass, is the principal organ for glucose disposal in the body (DeFronzo et al., 1985).

1.1.2 Insulin

Insulin is a hormone that is essential for regulating energy storage and glucose metabolism in the body (Pessin and Saltiel, 2000). Insulin stimulates liver, muscle, and adipose tissues and other insulin-sensitive cells to take up glucose from blood to be stored as glycogen in liver and muscle.

Insulin is secreted by the pancreatic β-cells in response to elevated levels of nutrients, such as increased circulating levels of glucose and amino acids after a meal. In liver, insulin decreases glycogenolysis and gluconeogenesis, thereby reducing hepatic glucose production (Sesti, 2006). In skeletal muscle and adipose tissue mainly insulin increases the rate of glucose uptake (DeFronzo and Tripathy, 2009, Roy et al., 1998).

The insulin receptor is composed of two extracellular α-subunits and two transmembrane β-subunits linked together by disulfide bonds to form an $\alpha_2\beta_2$ complex (Kahn and White, 1988, Yarden and Ullrich, 1988, Sesti et al., 2001, Van Obberghen, 1994). Binding of insulin to the α-subunits induces a signal across the plasma membrane that leads to conformational change of the receptor, resulting in autophosphorylation of several tyrosine residues within the β-subunits. The receptor then undergoes a series of intramolecular transphosphorylations.

The activated insulin receptor phosphorylates several substrates on tyrosine residues, including members of the insulin receptor substrate family (IRS1/2/3/4) (White, 1997, Patti and Kahn, 1998, Sesti et al., 2001). This further triggers phosphorylation of phosphatidylinositol 3-kinase (PI3K). The activation of PI3K leads to activation of Akt (also known as protein kinase B, PKB) at different sites (serine/ threonine) (Van Obberghen et al., 2001, Krook et al., 1997). Akt activation leads to translocation of insulin-responsive glucose transporter 4 (GLUT4) vesicles from their intracellular pool to the plasma membrane, and consequently increased glucose uptake into cells (Lanner et al., 2008).

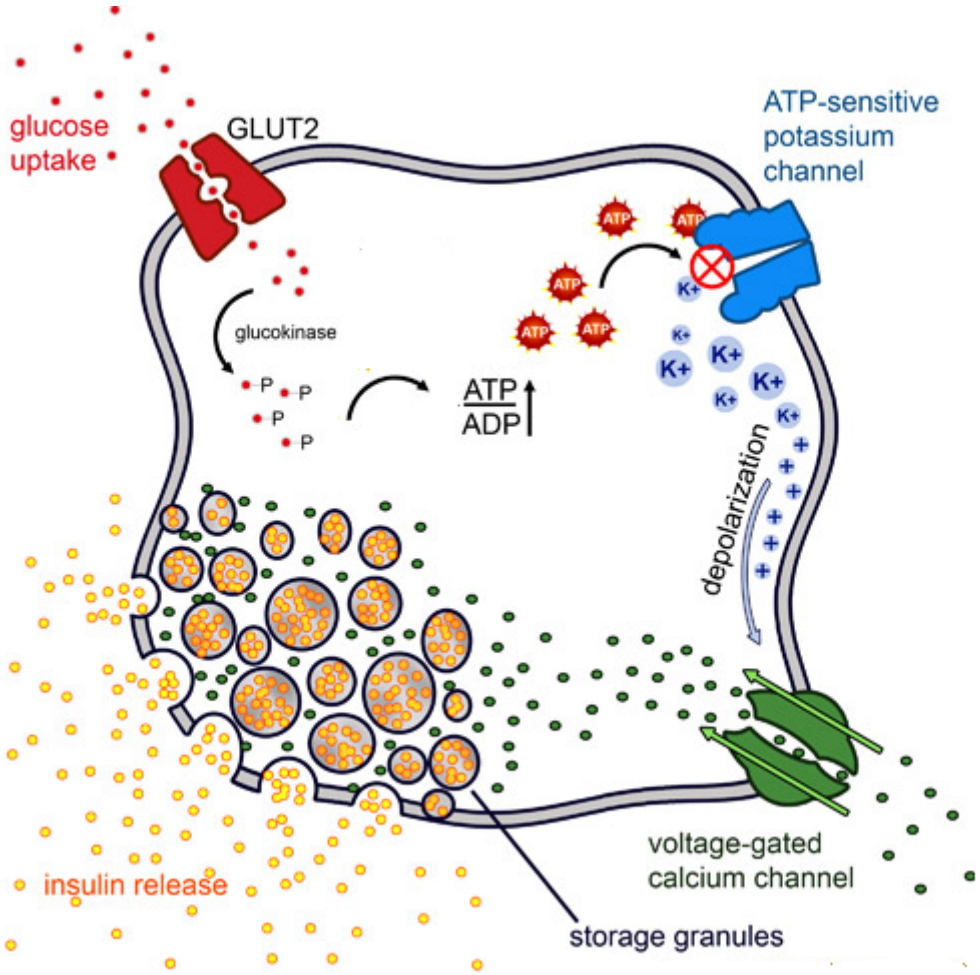
1.1.2.1 Insulin resistance (IR)

IR is a condition in which the cells of the body become resistant to the effects of insulin, causing physiological insulin levels to be insufficient to regulate glucose homeostasis by peripheral target tissues (Petersen and Shulman, 2002). As a result, a higher amount of insulin than normal is needed to achieve a sufficient insulin response. IR has been associated with conditions such as cardiovascular disease (CVD), hypertension, type 2 diabetes (T2D), obesity, and nonalcoholic fatty liver disease (Cohn et al., 2005).
IR in classic insulin target tissues (e.g, liver, skeletal muscle and adipose tissue) results in perturbation of body glucose homeostasis. IR in liver leads to reduced glycogen synthesis and storage and a failure to suppress glucose output into the blood (Kim et al., 2008). In extra-hepatic tissues, IR reduces glucose uptake and the storage of glucose as glycogen and triglycerides, respectively (Petersen and Shulman, 2002).
Initially β-cells compensate for IR by producing additional amounts of insulin, so that normal glucose tolerance is maintained (Purrello and Rabuazzo, 2000). This results in a state of hyperinsulinemia. Despite elevated insulin levels, as IR worsens, increased insulin secretion from the β-cells becomes less effective at lowering plasma glucose levels. Eventually, the secretion of insulin reaches a point at which it is unable to counteract the IR level, and subsequently hyperglycemia develops (Petersen and Shulman, 2002).

1.1.2.2 Insulin secretion

Insulin is synthesized in β-cells and stored in the cytosol in secretory granules. A rise in plasma glucose is the most important stimulus for insulin secretion. Glucose is transported into the β-cells by facilitated diffusion through the glucose transporter -2 (GLUT2) (Fig. 1). Glucose is metabolized, with subsequent production of ATP. An increase in cytoplasmic ATP/ADP ratio leads to the closure of ATP-sensitive potassium channels, causing depolarization of the plasma membrane. This in turn leads to the opening of voltage-gated calcium channels and an influx of extracellular calcium into the cell. An increase of cytoplasmic levels of Ca^{2+} causes vesicles containing insulin to be released from the β-cells by exocytosis (Ashcroft et al., 1994).

Fig. 1: Schematic model of the glucose induced insulin secretion in pancreatic β-cells.



1.1.3 Glucose transporters

Glucose cannot diffuse directly into cells, but enters by one of two glucose transporter mechanisms, Na^+-dependent-glucose transporters (SGLTs) and Na^+-independent glucose transporters (GLUTs). GLUTs are widely distributed in nearly all cells. GLUT1 is found in the brain and erythrocytes, GLUT2 is primarily in pancreatic β-cells, the liver and the kidneys, GLUT3 in the brain, and GLUT4 is found in heart, skeletal muscle and adipose tissue (Thorens and Mueckler, 2010). GLUTs exist in cell membranes in two conformational states. Glucose binding to the extracellular form alters its conformation and permits transport of glucose across the cell membrane. Glucose movement then follows a concentration gradient from high to low concentrations, so-called facilitated diffusion.

1.1.4 Liver in glucose homeostasis

The liver is a unique organ in keeping the balance between glucose release and uptake. In the fasted state, the liver is the primary producer of endogenous glucose , whereas in the fed state, elevations in circulating glucose and insulin stimulate the liver to take up

and store glucose (Edgerton et al., 2009). Therefore, the liver plays an essential role in maintaining glucose homeostasis.
Glucose is produced in the liver through two processes: glycogenolysis, from glycogen stores, and gluconeogenesis (Edgerton et al., 2009). In the fasting state, endogenous glucose production is necessary to keep the plasma glucose levels constant. The liver is responsible for about 80% of endogenous glucose production to the circulation in the fasting state (Cano, 2002, Bischof et al., 2001). Glycogenolysis and gluconeogenesis are partly under the control of glucagon. Glycogenolysis produces glucose during the first 8–12 hours of fasting. Over longer period of fasting, when glycogen in the liver is depleted, the liver switches to gluconeogenesis to maintain glucose levels.

In the fed state, insulin suppresses hepatic glucose production directly by its interaction with its hepatic receptors promoting glycogen synthesis by stimulating glycogen synthase and inhibiting glycogen phosphorylase (Edgerton et al., 2009).

In addition, insulin suppresses the release of gluconeogenic substrates from extrasplanchnic tissues and inhibits glucagon secretion from pancreatic α-cells, leading to cessation of glucose production via glycogenolysis and gluconeogenesis (Cherrington et al., 2007, Ballian and Brunicardi, 2007, Ader and Bergman, 1990, Giacca et al., 1992). Thus during both fasting and feeding, decreased hepatic insulin sensitivity results in dysregulation of hepatic glucose metabolism with impairments in fasting glucose levels and glucose tolerance such as in T2D.

Hepatic IR is accompanied by elevated basal hepatic glucose production that leads to fasting hyperglycemia (Consoli, 1992, Radziuk and Pye, 2002). On the other hand, in postprandial state hepatic IR leads to diminished suppression of hepatic glucose production and glucose clearance which further contribute to impaired glucose tolerance and postprandial hyperglycemia (Wahren and Ekberg, 2007). The rate at which the liver produces glucose during fasting, and the degree to which it takes up glucose following a meal, are determinants of glucose homeostasis. Thus, hyperglycemia associated with impairments in the regulation of fasting glucose production and glucose tolerance may be improved by therapeutics which target IR in the liver (Edgerton et al., 2009).

1.1.5 Skeletal muscle in glucose hemostasis

Skeletal muscle, due to its large mass (Shearer and Graham, 2002, Ehrenborg and Krook, 2009) is the primary site for glucose disposal in the body, such that up to 75% of a glucose load is converted to glycogen (Saltiel and Kahn, 2001, Jue et al., 1989). Glucose uptake into skeletal muscle provides cells with an important energy substrate and has a major impact on body glucose homeostasis.

The muscle uses energy to drive all the processes in order for the muscle to contract. Glucose and lipids are the main oxidative fuel substrate in muscle (Frayn, 2003).
At rest, glucose is the main energy substrate for skeletal muscle in the fed state. Storage of glucose as glycogen is enhanced by insulin stimulation of glucose uptake in muscle, via the increased the translocation of intracellular vesicles containing GLUT4 to the cell surface (Tremblay et al., 2003, Jessen and Goodyear, 2005, Ryder et al., 2001). Under fasting conditions, plasma glucose and insulin concentrations are low, leading to an increase in non-esterified fatty acid (NEFA) concentrations; thus fatty acids (FA) are then the main energy substrate for skeletal muscle. During exercise or muscle

contraction, glucose uptake increases, as a result of activation of AMP-activated protein kinase (AMPK) as well as increased intracellular Ca^{2+} levels (Hutchinson et al., 2008). The latter is also able to stimulate glucose uptake independently of contraction (Youn et al., 1991).
Skeletal muscle is also a primary site of IR in the context of metabolic disease, in particular, T2D and obesity (Bouzakri et al., 2005).

1.2 TYPE 2 DIABETES

T2D is a common disease involving multiple organs with dysregulation in glucose and lipid metabolism. It is a heterogeneous disorder characterized by chronic hyperglycemia due to impaired insulin secretion and insulin sensitivity. Noticeably, either IR or impaired β-cell function alone will not lead to the development of T2D (Ostenson, 2001). Rather, to develop T2D both IR and impaired β-cell should exist (Fig 2).

The incidence of diabetes is rapidly increasing worldwide and is estimated to be 285 million in 2010, and will exceed 400 million in 2030 (Shaw et al., 2010). Of these patients, almost 90% are accounted for by T2D (Nolan et al., 2006).

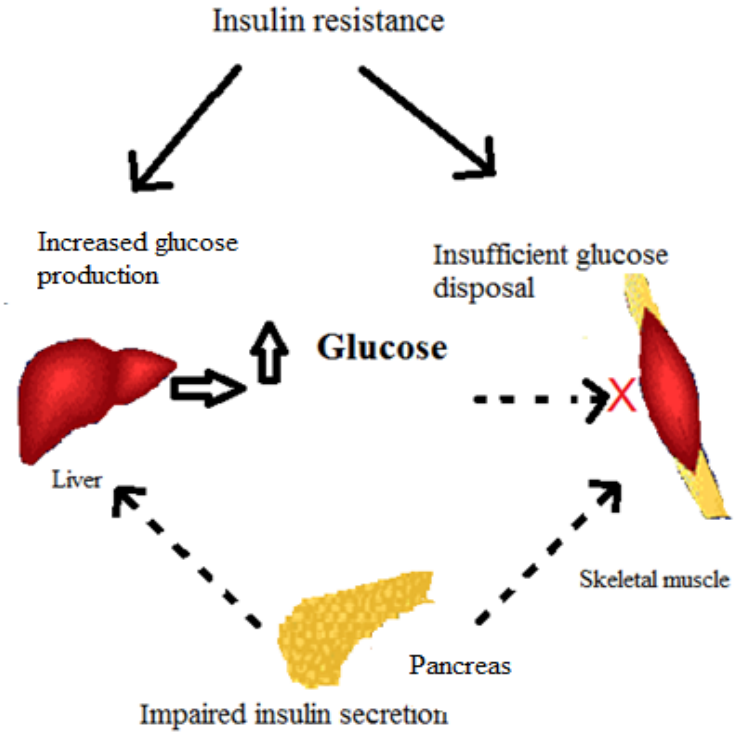
Available treatments for T2D moderate the glycemia, but fail to completely normalize metabolism. The long-standing elevation of plasma glucose as well as dyslipidemia is associated with chronic diabetes complications. Currently available therapies for T2D include sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones, glucagon-like peptide-1 (GLP-1) analogues, dipeptidyl peptidase-IV (DPP-IV) inhibitors and insulin. Patients treated with monotherapy often progress to combination therapy to obtain better control, and many patients eventually require insulin therapy (DeFronzo, 1999).

Sulfonylureas (SUs) increase insulin secretion by closing the potassium channels in β-cell membranes, leading to membrane depolarization, opening of voltage-dependent calcium channels and finally exocytosis of insulin. In addition to the side effects of SUs, which includeincreasedweight and a risk for hypoglycaemia, many patients develop secondary failure to SU action after 5-10 years. Non-SU insulin secretagogues, such as meglitinides (repaglinide and nateglinide) act via a mechanism similar to SU but possess shorter therapeutic duration. Biguanides (metformin) inhibit gluconeogenesis, and may also enhance extrahepatic glucose uptake via stimulation of AMP kinase (Wiernsperger and Bailey, 1999). α-Glucosidase inhibitors decrease glucose uptake from the intestine by inhibiting the degradation of polysaccharides to monosaccharides .

Thiazolidinediones (TZDs) act as agonists for the peroxisome proliferator activated receptor (PPAR) γ, and increase peripheral glucose uptake. Moreover, GLP-1 analogues (i.e. exenatide and liraglutide), and also DPPIV inhibitors (i.e. sitagliptin and vildagliptin) exert several effects, such as glucose-dependent stimulation of insulin release, suppression of glucagon release, slowdown of gastric emptying and reduction of food intake (Nauck, 1998, Perfetti and Merkel, 2000).

The number of therapies available for the treatment of T2D has increased. However, there are still difficulties in reaching the goal of normalizing glycemia without side effects. Hence, there is a great medical need to develop novel drugs that are both effective and which are free of side effects.

Fig. 2: Impairment of insulin secretion and insulin sensitivity in T2D.



1.2.1 Novel factor modulating glucose homeostasis

1.2.1.1 Gynostemma pentaphyllum (GP)

Gynostemma pentaphyllum (GP) *Makino-Cucurbitaceae* is a medicinal herb that has been widely used in Vietnamese and some other Asian countries;it has also been advertised in some European countries as a herbal tea. GP extract reportedly has numerous benefits, such as cholesterol-lowering, immune-potentiating, anti-tumor, anti-oxidant, and anti-diabetic effects (Li et al., 1993, Lin et al., 1993, Zhou et al., 1998, Jang et al., 2001).

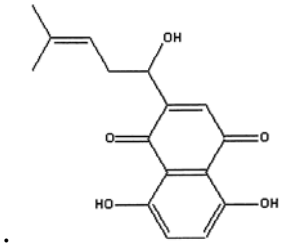
An extract of GP has been shown to decrease plasma glucose levels in mice and rats. From GP extract we purified and characterized an active substance, phanoside, that was demonstrated to stimulate insulin secretion in islets from healthy and diabetic rats and rats *in vivo* (Norberg et al., 2004, Hoa et al., 2007). Moreover, in a randomized placebo-controlledclinical trial in drug-naïve patients with T2D, GP extract significantly improved HbA1c values and fasting plasma glucose levels (Huyen et al., 2010). In addition, Homeostasis Model of Assessment –Insulin Resistance (HOMA-IR) studies indicated that the main effect was accomplished by improved insulin sensitivity.

1.2.1.2 Shikonin

Shikonin is a substance that is extracted from the dried gromwell root of the Chinese plant *Lithospermum erythrorhizon* (LE) (Fig 3). LE has been used in traditional Chinese medicine to treat a variety of disorders. As its genitor plant, shikonin was reported to possess numerous pharmacological properties, including anti-inflammatory

and antitumor properties and the ability to promote wound healing activity (Chen et al., 2002, Long et al., 2011). Shikonin has also been shown to increase glucose uptake in adipose tissues by augmenting insulin signaling (Kamei et al., 2002).

Fig.3
Chemical structure of shikonin (C16H16O5; molecular weight, 288.3 (Chen et al., 2003).



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1.2.1.3 ARA290 (Erythropoietin analogue)

ARA290 is a non-hematopoietic erythropoietin (EPO), analogue that consists of 11 amino acids. EPO is a well-established stimulant of red blood cell formation and survival, has been shown to exert tissue protective effects (anti-inflammatory and anti-apoptotic) (Choi et al., 2010). Erythropoietin receptor (EPO-R) is present in erythroid cells as well as in other non-erythroid tissues, including pancreatic islets (Fenjves et al., 2003). EPO treatment was shown to protect against diabetes development in type 1 and type 2 diabetic animal models by effects within the islets (Choi et al., 2010). However treatment with EPO is associated with an increased hematocrit that lead to an increased risk for the development of thrombosis. The EPO analogue, ARA290 (Brines et al., 2008), which lacks such a hematopoietic action, binds to the EPO-R –β-common receptor and protects a number of tissues in response to injury.

1.3 GOTO-KAKIZAKI (GK) RATS

The Goto-Kakizaki (GK) rat is a model of hereditary type 2 diabetes developed by repeated selective breeding of normal Wistar (W) rats, based on selecting male and female W rats with the highest glucose levels within the normal range during a glucose tolerance test (Goto et al., 1976, Yagihashi et al., 1978). Repetition of the selective breeding over numerous generations resulted in the production of non-obese diabetic rats from W rats, named Goto-Kakizaki rats. Colonies in Paris, Stockholm, Seattle and other sites were then initiated with breeding pairs from Japan (Metz et al., 1999, Lewis et al., 1996, Portha et al., 1991, Ostenson et al., 1993c). The Stockholm rat colony was initiated from progenitors delivered from the F40 generation of the original colony. The GK rat develops hyperglycemia post-natally and maintains moderately enhanced plasma glucose levels during its lifetime. In common with in human T2D, glucose intolerance in the GK rat is due partly to impaired insulin secretion, but also to IR in target tissues.

In these animals, impaired insulin release appears in early life and seems to be the primary defect (Abdel-Halim et al., 1995, Miralles and Portha, 2001). IR in skeletal muscle and liver is moderate and is most likely secondary to hyperglycemia, at least in case of the muscle (Bisbis et al., 1993, Abdel-Halim et al., 1995, Krook et al., 1997) . In GK rats, impaired glucose stimulated insulin secretion has been demonstrated *in vivo* (Gauguier et al., 1996, Salehi et al., 1999, Portha et al., 1991), in isolated pancreatic

islets (Hughes et al., 1994, Ostenson et al., 1993b), and in the perfused isolated pancreas (Abdel-Halim et al., 1996, Portha et al., 1991, Ostenson et al., 1993b). In GK rats from our colony, pancreatic insulin content and β-cells density are normal (Portha et al., 1991, Abdel-Halim et al., 1995, Abdel-Halim et al., 1994). The defective insulin response to glucose in GK rats is accompanied by a number of abnormalities in islet glucose metabolism, e.g. increased glucose cycling and increased glucose utilization (Hughes et al., 1994, Giroix et al., 1993, Sener et al., 1993, Ostenson et al., 1993b), decreased FAD-linked glycerol phosphate dehydrogenase and pyruvate carboxylase activities (MacDonald et al., 1996, Ostenson et al., 1993a) and decreased pyruvate dehydrogenase activity (Zhou et al., 1995).

GLUT2 is found to be underexpressed, but not to the degree that could account for the impairment of insulin release (Ohneda et al., 1993). This hypothesis is supported by the fact that glucokinase/hexokinase activities were found to be normal (Tsuura et al., 1993, Ostenson et al., 1993a). In addition, glycolysis rates in GK rat islets are unchanged or increased compared with control Wistar rat islets (Hughes et al., 1994, Ling et al., 1998, Giroix et al., 1993). Moreover, oxidation of glucose has been reported to be unchanged (Hughes et al., 1994, Sener et al., 1993, Ostenson et al., 1993b) .
In GK rats, hepatic IR is evident by an increase in hepatic glucose output (HGO) in connection with a dysregulation of hepatic fructose-2-6-bisphosphate. This hepatic IR is characterized by decrease in insulin receptor number but normal tyrosine kinase activity (Bisbis et al., 1993).

In the skeletal muscle, a defective activation of glucose accumulation into glycogen, possibly due to chronic activation of protein kinase C (Villar-Palasi and Farese, 1994, Avignon et al., 1996) has been suggested to contribute to IR and hyperglycemia in GK rats. Additionally, defective post-receptor signaling was characterized by alterations in insulin-stimulated glucose transport and PI-3K-activated Akt kinase (Krook et al., 1997).

The GK rat is regarded as one of the most relevant rodent models of non-obese T2D and has been widely used in experimental diabetes research. In this thesis **(Papers I, II** and **III)**, GK rats were used to observe and investigate factors modulating glucose homeostasis. The GK rats used were bred at this department (Department of Molecular Medicine and Surgery (Karolinska Institute, Stockholm, Sweden)

1.4 SEX DIFFERENCES IN METABOLISM

Sex difference is an important variable that should be considered in all areas of health-related research (Tingen et al., 2010). It is well established that body size and body composition are sexually dimorphic (Rogol et al., 2002, Roemmich et al., 2002). Skeletal muscle mass is larger in males than in females (Janssen et al., 2000), while females have a higher percentage of fat mass than males (Wu and O'Sullivan, 2011, Loomba-Albrecht and Styne, 2009); this is characterized by a higher proportion of body fat in the gluteal-femoral region, whereas males have more body fat in the abdominal (visceral) region (Geer and Shen, 2009). Fat within the visceral area is associated with a statistically higher risk of hyperlipidemia, hypertension, IR (Hirosumi et al., 2002), and T2D (Duman et al., 2003).

The prevalence of abnormalities of glucose metabolism has been estimated to be higher in middle-aged men than in age-matched women in (Kuhl et al., 2005) which might

indicate that sex dependent hormones play a role in this context. This notion is supported by animal studies showing that estrogen receptor knockout mice develop fatty liver, hepatic IR and impaired glucose tolerance (Kaye et al., 2006). Based on previous studies (Tarnopolsky and Ruby, 2001), males might be predicted to more rapidly mobilize hepatic carbohydrates as compared to females. Moreover, when estrogen levels decrease, for example in the menopausal state, inter-sex differences decrease and women develop an increase in visceral fat (Goodpaster et al., 2005, Nicklas et al., 2004).

2 AIMS

The general aim of this thesis was to investigate factors modulating glucose homeostasis in healthy and diabetic rats.

Specific aims in the individual papers were:

- To elucidate the direct effect of an extract of a Southeast-Asian herb *Gynostemma pentaphyllum* (GP) on hepatic glucose output in spontaneously type 2 diabetic Goto-Kakizaki (GK) rats.

- To explore the effects of shikonin, a naphthoquinone derived from a Chinese plant, on total body glucose homeostasis in GK rats, and to investigate the effects of shikonin on glucose uptake and the mechanisms of its action in L6 skeletal muscle cells.

- To study the effect of a non-hematopoietic erythropoietin analogue ARA290 on glucose homeostasis in GK and Wistar rats and to explore the mechanism of action that underlies the anti-diabetic effects of ARA290 in GK rats.

- To investigate sex differences in carbohydrate and lipid metabolism in the rat liver.

3 MATERIALS AND METHODS

This section gives a general commentary of some of the methods central to the work presented in this thesis. More detailed descriptions of these and other methods used can be found in the corresponding papers.

3.1 ANIMALS

3.1.1 Wistar rats (W)

Normal Wistar rats (W) were purchased from a commercial breeder (Scanbur BK, Sollentuna, Sweden) and used as non-diabetic controls **(Paper III)**.

3.1.2 Sprague-Dawley rats (SD)

Male and female rats Sprague-Dawley rats (SD) rats were purchased from a commercial breeder (Scanbur BK) **(Paper IV)**.

3.1.3 Goto-Kakizaki rats (GK)

The Goto-Kakizaki (GK) rat is a model of hereditary type 2 diabetes developed by selective breeding of normal Wistar (W) rats with a slight impairment of glucose tolerance. Repetition of the selective breeding resulted in rats exhibiting spontaneous non-obese diabetes. The GK rat develops hyperglycemia post-natally and maintains moderately enhanced plasma glucose levels during its lifetime. In common with human T2D, glucose intolerance in the GK rat is due partly to impaired insulin secretion, but also to IR in target tissues.
In this thesis **(Papers I, II** and **III)** GK rats were studied and investigated for factors modulating glucose homeostasis. The GK rats used were bred at our department (Department of Molecular Medicine and Surgery, Karolinska Institute, Stockholm, Sweden).
All animal experiments were approved by the regional Ethics Committee on Animal Experiments.

3.2 TREATMENTS

3.2.1 *Gynostemma pentaphyllum* (GP)

The GP extract used in our study was produced by from whole GP plants, collected by the Institute Materia Medica (Hanoi, Vietnam) from the Vietnamese mountains. Plant extract were prepared by boiling in water for 2 hours, after which concentrated ethanol (70%) was added to remove impurities by distillation at low pressure. The resulted material was ground into a powder to form soluble tea particles.
In paper I, we investigated GP's anti-diabetic effects in GK rats, and explored the observed improvement in hepatic insulin sensitivity. GP extract or green tea placebo were administrated orally at a dose of 800 mg/kg b.w. to unanesthetized GK rats by gavage through an enteral feeding tube (polyvinyl chloride, sterile VYCON, Lab. Pharmaceutiques Vycon, Ecouen, France). The administrations were performed twice daily for three days or three weeks.

3.2.2 Shikonin

In paper II, we examined the effect of shikonin (Interchem, NJ, USA) on glucose uptake in skeletal muscle cells *in vitro*, and on total body glucose homeostasis in diabetic Goto-Kakizaki rats.In the latter *in vivo* experiments, intraperitoneal (i.p.) vehicle (DMSO/olive oil) (9:1), was administrated initially once daily for 4 days. One week after vehicle administration, shikonin were similarly administered to the same GK rats at a dose of 10 mg/kg b.w.

3.2.3 ARA290 (Erythropoietin analogue)

In paper III, we studied the effects of ARA290 on different aspects of glucose homeostasis in GK rats and non-diabetic control rats. ARA290 at a dose of 30 µg/kg b.w., or placebo were administered s.c. to GK and matched Wistar (W) rats once daily for four weeks.

3.3 TOLERANCE TESTS

3.3.1 Intraperitoneal glucose tolerance test (IPGTT) and insulin response

Intraperitoneal (i.p.) glucose tolerance tests (IPGTTs) were performed in overnight-fasted rats. Blood samples for determination of glucose (about 20µl/sample) were taken after a small tail incision. Plasma glucose concentrations were measured by glucometer (Accu-Chek Aviva) at 0, 15, 30, 60, and 120 minutes after an i.p. injection of glucose (2 mg/g body weight; Glukos APL 500 mg/ml) **(Paper I)**. In addition, in paper III, blood samples were collected at 0 and 30 min during the IPGTT for insulin analyses using radioimmunoassay (Herbert et al., 1965).

3.3.2 Intraperitoneal pyruvate tolerance test (IPPTT)

Intraperitoneal pyruvate tolerance tests (IPPTTs) was carried out in overnight-fasted GK rats. Plasma glucose concentrations were obtained at 0, 15, 30, 60, and 120 minutes after an i.p. injection of pyruvate (2 mg/g body weight; sodium pyruvate, SIGMA) **(Papers I** and **III)**.

3.3.3 Subcutaneous (s.c.) insulin tolerance test (SCITT)

For the SCITT, insulin was injected i.p. at a dose of 0.5 U/kg in overnight-fasted **(Papers I** and **III)** and non-fasted **(Paper II)** GK rats. Plasma glucose levels were measured before the injection of insulin at 0 min, and then every 15 minutes for 2 hours and every 30 minutes for further 2 hours.

3.4 *IN SITU* PERFUSION OF RAT LIVER

Liver perfusion *in situ* is a valuable technique for the study of hepatic metabolism. Rats were anesthetized with an intraperitoneal injection of ketamine (Pfizer AB, Täby, Sweden) at a dose of 60-70 µg/g b.w. Subsequently, rats under went median laparotomy and the portal vein was identified and cannulated. Thoracotomy was then performed, and a second cannula was placed in the inferior vena cava via the right atrium. Rat livers were perfused *in situ* without recirculation via the portal vein using Krebs-Henseleit bicarbonate buffer (118.4 mM NaCl, 4.7 mM KCl, 1.9mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 25 mM $NaHCO_3$, equilibrated with 95% O_2 and 5% CO_2, pH 7.4) with 0.2% bovine serum albumin (BSA), and 10 mM HEPES (Doi et al.,

2001) in a 37°C cabinet. The perfusion pressure was constant with a flow rate of 3.0-4.0 ml/min/g liver under basal conditions. Perfusate was drained from the inferior vena cava. Samples were collected every 2 min after a brief period of initial perfusion to completely remove blood from the liver. Perfusion then was continued for a further 8-15 min **(Papers I** and **IV)**. In paper I, hepatic glucose production was stimulated by adrenalin (Merck AB NM, Stockholm, Sweden) which was added into the perfusion medium (KRB) to a final concentration of 50 nM. The glucose levels in the perfusate were measured by a Glucometer (YSI 2300, STST PLUS, VWR).

3.5 ISOLATED PANCREATIC ISLETS

3.5.1 Insulin secretion (Paper III)

After 4 weeks of treatment with either ARA290 or placebo, GK rats were sacrificed by decapitation. Islets were isolated by collagenase digestion of the pancreas (Hoa et al., 2004). Directly after isolation, islets were incubated to study glucose-induced insulin release. After a pre-incubation period of 30 min at 37°C in Krebs-Ringer bicarbonate buffer solution (KRB), supplemented with 2 mg/ml bovine albumin, 10 mM HEPES and 3.3 mM glucose, pH 7.4, batches of three islets were incubated for 60 min at 37°C in 200 µl of KRB as above, and with either 3.3 or 16.7 mM glucose. After incubations, aliquots of the media were taken for radioimmunoassay of insulin.

3.5.2 Intracellular levels of Ca^{2+} concentration $[Ca^{2+}]_i$ in islets (Paper III)

Islets were incubated with 2 µM fura-2 AM and changes in $[Ca^{2+}]_i$, i.e. the fluorescence ratio at 355/380 nm, were analyzed on a microscope consisting of a Zeiss Axiovert 200M with a fluorescence imaging system, using Andor iQ software with an Andor iXon DV887DCS-BV camera and a Cairn monochromator for excitation (Andor Technology plc, Belfast, UK) (Healy et al., 2010, Yang and Gillis, 2004).

3.6 GLYCOSYLATED HAEMOGLOBIN (HBA1C) MEASUREMENTS

Red blood cells were lysed and the proteins desalted using 100 µL OMIX C4 tips (Agilent Technologies, Santa Clara, CA), using the manufacturer's protocol, with 10 loading cycles and elution with 10 µL 75% MeOH. Mass spectra were acquired on a 15 tesla solariX Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany). After desalting, 5 µL of each tip eluate was diluted with 95 µL H_2O: MeOH 1:1 (v/v) and 0.1% formic acid and infused directly into the electrospray source at 2 µL/min using the built-in syringe pump and a 100 µL syringe. For HbA1c quantitation, 64 full profile spectra of 1 M data points (1.9 s transient) covering *m*/*z* 829-1,000 were averaged, zero-filled (1X) and sine square apodized (Marshall et al., 1998). Quadruple transmission was optimized at the mid-point of the *m*/*z* range. The resolving power (*m*/Δ*m*, FWHM) of the hemoglobin chains was ~150,000. Wider range spectra (*m*/*z* 598-2,000) were also acquired to characterize the protein contents of the sample and the charge state distributions of the different hemoglobin chains. All spectra were acquired in broadband mode and externally calibrated using the heme monomer and dimer peaks.
After conversion of full profile data to mzXML (Pedrioli et al., 2004), Xtractor (Selman et al., 2010) as used to extract the area under the six most abundant isotopic peaks of the $[M+18H]^{18+}$ α-chain (UniProtKB HBA_RAT, *m*/*z* ~845.3), β-chain (UniProtKB HBB1_RAT, putatively with the T124S mutation, *m*/*z* ~880.6), the same β-chain with a glucose adduct (*m*/*z* ~889.6) and corresponding background signal from an empty

region of the spectra. The fraction of glucose adduct was reported as the ratio of the signal of the β-chain with a glucose adduct to the total β-chain signal.

3.7 HEMATOCRIT MEASUREMENTS

Blood samples were collected in EDTA-anticoagulated tubes, and hematocrit was measured using an automated blood analyzer (Scandivet AB, Enköping, Sweden).

3.8 EXPRESSION PROFILING USING MICROARRAYS

The microarray is a multiplex technology used to determine transcript profile. In paper IV, microarrays containing 70 mer oligonucleotide probes for 27 649 rat protein-coding genes were fabricated and used to obtain transcript profiles. Total RNA from each sample wasDNase-treated using RNeasy MiniElute Cleanup kits (QIAGEN), and RNA concentrations measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). The microarray experiments were performed using the Pronto!TM Plus Direct Systems (Corning Incorporated and Promega Corporation, NY), according to the manufacturer's instructions using 5 µg total RNA from each sample. Samples from male rat livers were hybridized with female samples on individual arrays to obtain a direct effect of sex on hepatic gene expression. Each hybridization compared Cy3-labeled cDNA reverse transcribed from RNA isolated from female rat livers with Cy5-labeled cDNA isolated from male livers. Each experiment was analysed using individual samples and dye-swopping (Stahlberg et al., 2004).
Identification of sex-dependent genes was performed usingSAM 1.21 (Significance Analysis for Microarray) software incorporated into Microsoft Office Excel. A 5% false discovery rate was used as a first cut-off. Genes with a greater than 1.5-fold difference between the sexes were considered as being sex-dependent. The results are presented as the mean of at least three independent determinations.

3.9 AKT AND AMPK PHOSPHORYLATION

Whole liver cell lysates were obtained by homogenizing 1 g of liver in 3 ml RIPA buffer. The resulting supernatants were collected and the degree of insulin signaling was analysed by measuring the degree of insulin-dependent phosphorylation of Akt. Akt activation was determined by analysing the amount of phosphorylated Akt (p-Akt-Ser473) in relation to total Akt, using commercially available ELISA kits (Biosource). The degree of AMPK phosphorylation was determined using antibodies detecting p-AMPK-Thr172 or AMPK (1:1000) from Cell Signaling. Densitometry analyses were performed using the software Quantity One 4.6.5 Basic (Bio-Rad Laboratories) to compare the amount of phosphorylated AMPK (p-AMPKThr172) in relation to total AMPK.

3.10 NMR SPECTROSCOPY

Liver perfusate samples (500 µl) were mixed with 50 µl standard solution of Hexa deutero-4, 4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA), purchased from Onyx Scientific Ltd (Sunderland, United Kingdom).
The NMR spectroscopic measurements were made on a Bruker 600 MHz instrument

(Bruker BioSpin, Rheinstetten, Germany) operating at 600.23 MHz, equipped with a 5 mm inverse probe and a SampleJet sample changer. 1H-NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence to attenuate broad signals arising from macromolecular components (Meiboom and Gill, 1958). Altogether 512 transients were acquired into 64 K data points using a spectral width of 12019 Hz with a spin echo loop time of 76.8 ms and relaxation delay of 2.0 s, with a total repetition time of 4.82 s. Suppression of the water resonance was achieved by presaturation during the relaxation delay and the spin-echo loop.

3.11 REAL–TIME PCR (RT-PCR)

The RT-PCR method permits amplification and quantification of a targeted DNA molecule. In paper IV, total RNA was treated with DNase (RQ1) and reverse transcribed using iScript™ reverse transcriptase and 5x buffer for First Strand cDNA synthesis (Bio-Rad Laboratories AB). The purity of the synthesized cDNA was checked by agarose gel electrophoresis. Each first strand cDNA served as a template in a 20 µl real-time PCR reaction mix containing the primers for the gene of interest and iQ SYBR Green Supermix (Bio-Rad Laboratories AB). Quantification of gene expression (Gustavsson et al., 2009) was performed according to the manufacturer's protocol using a DNA Engine Opticon™2 real-time PCR detection system (MJ Research).

The protocol was validated for each gene of interest by checking melting curves for the absence of primer–dimers and other unwanted amplicons.

The level of individual mRNAs was normalized with the level of the housekeeping gene ribosomal protein long-chain protein Pθ (RPLP).

3.12 WESTERN BLOT

Western blotting is a technique to identify proteins with the help of specific antibodies. This method was used to analyze proteins from L6 cells **(Paper II)**.

Cells were serum-starved overnight before each experiment and exposed to drugs for different times and at different concentrations (Lindquist et al., 2000). After 2 h of shikonin or insulin stimulation, the L6-cells were lysed directly in the well by the addition of 80 µl 65°C SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue). Cells were scraped, transferred to an Eppendorf tube on ice, sonicated for 10 s and heating to 100°C for 5 min.

Aliquots of the samples were separated on a 12% polyacrylamide gel and electrotransferred to a Hybond-P PVDF membrane (pore size 0.45 µm; Amersham Pharmacia Biotech) with a semidry electroblotter . After transfer, the membranes were allowed to soak in Tris-buffered saline for 5 min, followed by quenching of nonspecific binding (1 h at room temperature in 5% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline).

Membranes were probed using AMPK, phospho-AMPK (Thr172), Akt, phospho-Akt (Ser473), or phospho-Akt (Thr308), (diluted 1:1000 except phospho-AMPK diluted 1:500) antibodies. Protein-antibody complexes were detected using a secondary antibody (HRP-linked anti-rabbit IgG, diluted 1:2000) and chemiluminescence (ECL,

Amersham Pharmacia Biotech). All antibodies were purchased from Cell Signalling. The bands were scanned and quantified by the software MacBiophotonics ImageJ.

3.13 L6 CELL CULTURE AND GLUCOSE UPTAKE

L6 myoblasts can be differentiated into myotubes and are commonly used for studies of glucose uptake. In paper II, L6 cells (from ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose, supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 2 mM L-Glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin in a 37°C incubator with 8% CO_2. Upon reaching confluence, differentiation was induced by media containing 2% (v/v) FBS for 7 days, with medium changes every second day. Differentiated L6 myotubes were treated with 1 µM shikonin or 1 µM insulin for 2 h or 20 h. Glucose uptake was measured using the 2-deoxy-[^{3}H] D-glucose method. Briefly, cells were serum-starved overnight before each experiment and glucose uptake was measured on day 7. The medium was changed to serum-free media the evening before the experiments, and changed to fresh medium on the morning 2 h or 20 h before cells were exposed to shikonin treatment. Cells were washed twice in warm PBS before a change of media, and drugs were placed in DMEM devoid of glucose for 10 min. 2-Deoxy-[^{3}H] D-glucose (50 nM) was added for 6-10 min at 37°C. The reactions were terminated by washing twice in ice-cold PBS. Cells were incubated in 0.2 M NaOH for 1 h at 60°C, and the samples were transferred to scintillation vials with scintillant and allowed to sit at room temperature for 1 h before being counted (Merlin et al., 2010, Hutchinson and Bengtsson, 2006).

3.14 ATP LEVEL MEASUREMENTS

L6-myotubes were serum-starved overnight and new medium was added for 2 h before stimulation with drugs for 2 h. Measurement of the AMP:ATP ratio was performed on day 7. One ml of boiling water was added to each well of a six-well plate, and the cells were scraped and boiled (3 min). Samples were centrifuged (12,000 g, 4°C, 10 min) to pellet cell debris, and the supernatant fractions were used for further analyses. Each sample was diluted 1:10, three aliquots (20 µl) were transferred to a white 96-well plate, and 30 µl of either reagent A (aqueous tricine buffer [40 mM, pH 7.8], $MgSO_4$ [8 mM], EDTA [0.17 mM], which measures total cellular ATP), reagent B (reagent A buffer supplemented with dCTP [0.1 mM], nucleoside-5′-diphosphate-kinase [10 U/ml], which converts the ADP in a given sample to ATP) or reagent C (reagent B supplemented with myokinase [10 U/ml], which converts the AMP and ADP in a given sample to ATP) added for measurement of ATP, ADP and AMP, respectively. Samples were incubated for 12 h at 37°C, after which the reactions were terminated by boiling for 5 min, and then 50 µl of luciferin–luciferase reagent (aqueous tricine buffer [25 mM, pH 7.8], $MgSO_4$ [5 mM], EDTA [0.1 mM], D-luciferin [0.5 mM], luciferase [10 µg/ml], dithiothreitol [2 mM], CoA [0.5 mM]) were added to each well. ATP levels were measured in duplicate using a commercial kit (ATP determination kit time stable assay; Biaffin, Kassel, Germany). Results are expressed as nanomoles ATP per milligram protein (Merlin et al., 2010, Hutchinson and Bengtsson, 2006).

3.15 OXYGEN CONSUMPTION

Oxygen consumption was measured in the absence or presence of 1 µM shikonin in differentiated L6 cells and skeletal muscle mitochondria. L6 cells were harvested by trypsin digestion, while mitochondria were isolated from mouse muscles (Shabalina et al., 2010). Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instruments Company, Yellow Springs OH, U.S.A.) in a sealed chamber at 37°C. L6 cells were added to a magnetically stirred oxygen electrode chamber containing DMEM in a final volume of 1.1 ml. The output signal from the oxygen electrode amplifier was collected by a PowerLab/ADInstrument (application program Chart v5.1.1.). The Chart data files were converted to absolute values, based on an oxygen content of 217 nmol of O_2 per ml of water and on the number of cells used. For calculation of stable oxygen consumption rates, mean values during 1 min were obtained from these recordings.

3.16 INTRACELLULAR LEVELS OF CA^{2+} CONCENTRATION $[CA^{2+}]_I$ IN L6 CELLS (PAPER II)

Cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured during exposure to shikonin (1 µM) or insulin (1 µM) using the fluorescent ratiometric Ca^{2+} indicator indo-1. Cells were loaded with indo-1 by exposing them to indo-1-AM (8.3 mM, Invitrogen) for 20 minutes, followed by at least 20 minutes of washing. Indo-1 was excited with light at 360 nm, and light emission at 405±5 and 495±5 nm was measured with two photomultiplier tubes. The ratio (R) of the light emission at 405 nm to that at 495 nm was converted to $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). After establishing a baseline, shikonin or insulin was added.

3.17 IMMUNOCYTOCHEMISTRY

L6 cells stably transfected with GLUT4myc were plated in 4 well culture chamber slides (BD Biosciences, Franklin Lakes, BJ). Cells were serum-starved 16 h before stimulation with insulin or shikonin. After stimulation, the cells were washed with PBS and fixed for 5 min with 4% formaldehyde in PBS, and quenched with 50 mM glycine in PBS for 10 min. Cells were blocked with 5% BSA in PBS for 1 h and incubated with Myc-tag primary antibody solution (1:200 dilution in 1.5% BSA in PBS) at 4 ^oC overnight, followed by washing with PBS and a 1 h incubation at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:500 dilution, 1.5% BSA in PBS). Slides were washed with PBS and mounted with ProLong Gold antifade reagent (Invitrogen). Images were observed in a confocal microscope, ZEISS LSM 510 META. Quantification of immunocytochemistry images was performed by using the ImageJ program (NIH).

3.18 STATISTICAL ANALYSIS

In **papers I, II** and **III**, the results have been calculated as mean ± SEM, and comparisons of the means have been performed by the unpaired Student´s t-test, ANOVA with the Tukey post hoc-test, or by the paired Student's t-test, using Bonferroni´s correction for multiple testing, as appropriate.

In **paper IV**, two-way ANOVA was performed to determine whether there were significant effects of sex, fasting or insulin treatment on measured variables, or significant interactions i) between sex and fasting, or ii) between sex and insulin. Subsequently, if the interaction was found to be significant, one-way ANOVA was conducted and multiple comparisons with Fisher's Least Significant Difference (LSD) test was employed to compare i) 4h-fasted males and females and the 12 h fasting effect, or ii) saline-treated males and females and the insulin effect in males and females. Friedman's non-parametric test was utilized to determine the effect of insulin treatment or a sex effect in ^{1}H-NMR metabolite data. Where indicated, groups were also compared using Student's t-test. P-values < 0.05 were considered significant.

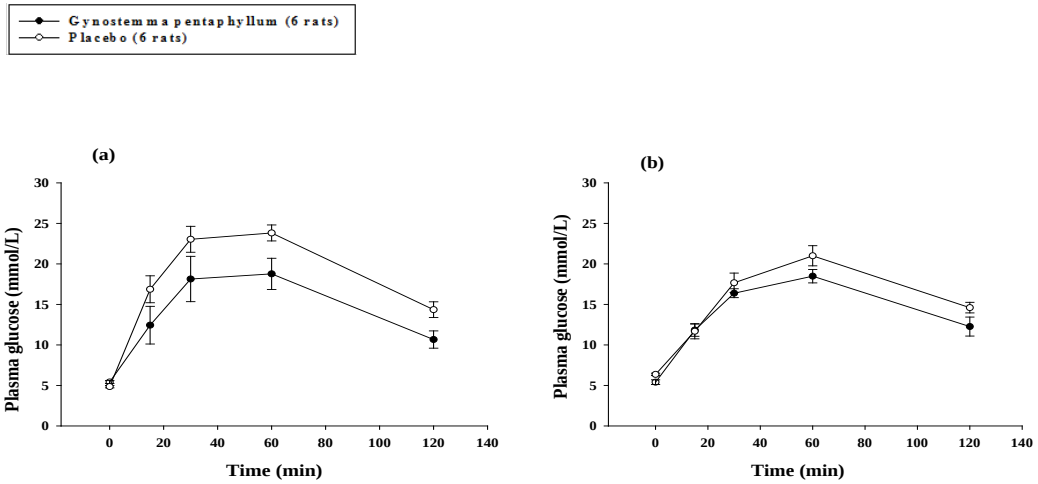
4 RESULTS & DISCUSSION

4.1 HERBAL EXTRACT OF *GYNOSTEMMA PENTAPHYLLUM* DECREASES HEPATIC GLUCOSE OUTPUT IN TYPE 2 DIABETIC GOTO-KAKIZAKI RATS (PAPER I)

In this paper, we investigated the effect of GP extract on modulating hepatic glucose production in GK rats treated orally with GP or placebo extract daily, during three days or three weeks. We found that the three-week, but not the three-day, treatment with GP reduced PG levels in GK rats ($p= 0.027$), whereas PG levels were not decreased after placebo treatment. After three weeks of treatment, glucose tolerance evaluated by an intra-peritoneal glucose tolerance test was improved in the GP-treated, compared to the placebo-treated, group (areas under the glucose curves, AUCs; $p = 0.013$; Fig.4a). In an intra-peritoneal pyruvate tolerance test, the glucose response from minute 15 to minute 120 was lower in the GP group ($p< 0.05$; Fig.4b).

In liver perfusions, the basal HGO in GP treated rats was 27% lower than that of the placebo group. Moreover, the AUCs for hepatic glucose output (HGO) during the first 18 min (0-18 min) were decreased in GP-treated rats, compared with placebo-treated rats ($p<0.05$).

Fig. 4: Mean of plasma glucose levels during IPGTT (a) and IPPTT (b) after three weeks of treatment of GK rats with GP.



In addition, the response to an infusion of 50 nM adrenaline after 8 min of the perfusion (8-18 min) increased HGO in all rats, although this effect of adrenalin tended to be suppressed in GP extract-treated rats, compared to placebo-treated rats. Three weeks of GP treatment significantly reduced hepatic glycogen content, but not glycogen synthase activity, compared to placebo group ($p<0.007$). GP has been reported to exert a variety of effects, such as anti-oxidant (Li et al., 1993), lipid lowering (la Cour et al., 1995), anti-inflammatory (Lin et al., 1993, Huang et al., 2007), anti-carcinogenic (Lu et al., 2008, Chen et al., 2006) and pro-apoptotic effects (Wang et al., 2002). In our previous investigations in type 2 diabetic patients, treatment with GP extract (Huyen et al., 2010) induced a potent anti-hyperglycemic effect. Insulin sensitivity was enhanced, as

demonstrated by reduced HOMA-IR, and fasting PG levels were significantly decreased after 12 weeks of treatment.

In our study, three days' treatment with GP extract did not change the PG levels, while long-term three-week treatment induced a significant decrease in PG levels, improvements in glucose tolerance, and reductions in HGO, compared to placebo-treated rats. However, in the insulin sensitivity test, which reflects insulin sensitivity mainly in extra-hepatic tissues, no differences were observed between the two groups, suggesting that the primary effect of GP extract is unlikely to be exerted via extra-hepatic tissues, i.e. mainly skeletal muscle. Therefore, these findings indicate that GP-induced improvements in glucose tolerance in GK rats is due to an effect in the liver, which may at least partly due to decreased HGO. Insulin decreases HGO by activating glycogen synthesis and glycolysis, and by suppressing gluconeogenesis (Cherrington et al., 2007). Glycogen is the intracellularly stored form of glucose, and its levels in various tissues, particularly in the liver and skeletal muscle, reflect the action of insulin in stimulating glycogen synthase and inhibiting glycogen phosphorylase (Edgerton et al., 2009). We have shown that hepatic glycogen synthase activity after three weeks of treatment did not differ between GP-treated and placebo-treated rats. Nevertheless, the hepatic glycogen content after GP extract treatment was significantly lower than that after placebo treatment. In the liver, is controlled by a number of different mechanisms. Phosphotyrosine phosphatase 1B (PTP1B) is known to negatively modulate insulin action on hepatic glucose metabolism through tyrosine dephosphorylation of the insulin receptor and/or insulin receptor substrates (Haj et al., 2005). Interestingly, recent experimental studies demonstrate that GP extract inhibits PTP1B activity, which may lead to enhanced insulin sensitivity and thereby improved glucose tolerance (Hung et al., 2009, Xu et al., 2010).

Furthermore, a recent study has shown that hepatic glycogen content was significantly reduced in PTP1B -/- transgenic mice, as compared to wild-type controls (Escriva et al., 2010). Therefore, it can be speculated that GP extract improves hepatic insulin sensitivity to some extent through inhibiting PTP1B. Since in the GP extract-treated GK rats the pyruvate tolerance test showed a decreased glucose response, it seems likely that the improvement in hepatic insulin sensitivity is partly accounted for by a reduction of gluconeogenesis.

In conclusion, the results of this study demonstrates that three-weeks' treatment of GP extract exerted anti-diabetic effects in GK rats, reducing plasma glucose levels and HGO, suggesting that GP improves the hepatic insulin sensitivity by suppressing gluconeogenesis.

4.2 SHIKONIN INCREASES GLUCOSE UPTAKE IN SKELETAL MUSCLE CELLS AND IMPROVES PLASMA GLUCOSE LEVELS IN DIABETIC GOTO-KAKIZAKI RATS (PAPER II)

In this paper, we examined the possible effect of shikonin on glucose uptake in skeletal muscle as well as on total body glucose homeostasis.

Our results revealed that stimulation with shikonin (1μM) for 2 h increased glucose uptake in L6-myotubes ($p<0.01$), a response which was of the same magnitude as that obtained following insulin treatment (1μM, $p<0.01$) (Fig. 5). Stimulation for 20 h with

shikonin or insulin at the same concentrations tended to induce increases in glucose uptake but the improvment was not statistically significant.

In L6 myotubes, shikonin treatment (1μM, 2 h) did not affect phosphorylation of Akt, whilst insulin treatment (1 μM, 2 h) increased phosphorylation of Akt at residues s473 or t308. Moreover, shikonin treatment (1 μM, 2 h) did not mimic the effect of the AMPK activator AICAR (2 mM, 2h), which increased AMPK phosphorylation at residue t172 by ~2 fold. Furthermore, shikonin did not induce any change in the AMP:ATP ratio. Addition of 1 μM shikonin increased oxygen consumption in intact L6 muscle cells. Shikonin had no effect on isolated skeletal muscle mitochondria, either in the presence or absence of the substrate pyruvate, indicating that shikonin does not act directly on mitochondria. Shikonin increased the intracellular levels of calcium in L6 cells. However, the cell-permeable calcium chelator, BAPTA-AM (5 μM), blocked shikonin-stimulated glucose uptake. Furthermore, we found that shikonin stimulated the translocation of GLUT4 from intracellular vesicles to the cell surface in L6 myotubes (Fig. 6).

Fig.5: Shikonin increases glucose uptake in L6 myotubes; cells were treated with 1μM shikonin (sh) or 1μM insulin for 2 h. Graph shows mean ± SEM for 7 rats ** P,0.01 ***P,0.001

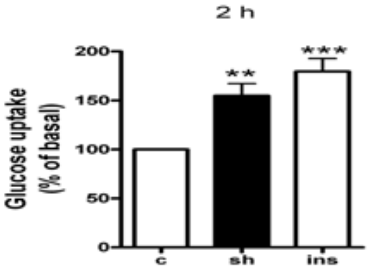
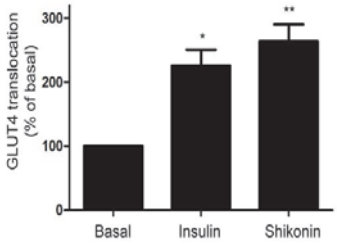


Fig.6: Shikonin stimulates translocation of GLUT 4 expressed as % of basal. Graph shows mean± SEM of 3 rats.



We also investigated the effect of shikonin on glucose homeostasis in diabetic GK rats. GK-rats were treated for 4 days with a daily i.p. injection of shikonin (10 mg/kg). In these rats, PG levels were significantly lowered on the 2nd and 4th days, compared to day 1 ($p < 0.01$ and $p<0.001$, respectively) (Fig. 7). Also, the areas under the glucose curve (AUCs) were significantly lower in shikonin-treated rats compared to control rats ($p=0.014$). In a s.c. insulin tolerance test (SCITT), administration of insulin reduced PG concentrations in all rats. The plasma glucose levels prior to insulin injection were lower in shikonin-treated rats compared to control rats ($p =0.007$) (Fig. 8). The total AUCs from 0 to 240 min were similar in shikonin-treated and control rats. However, for the period corresponding to 30-240 min after injection of insulin, the AUCs were lower in shikonin-treated rats, compared to control rats ($p<0.02$).

Shikonin is a naphtoquinone derivative from the plant *Lithospermum erythrorhizon* that inhibits the formation of the NADPH oxidase (Pageorgiou V.P et al., 1999). The compound has been shown to stimulate glucose uptake in 3T3-L1 adipocytes, primary rat adipocytes and cardiomyocytes (Kamei et al., 2002).

In L6 myotubes, shikonin and insulin increase glucose uptake to the same extent.

However, unlike insulin, shikonin did not affect Akt-phosphorylation, suggesting that in L6 myotubes, shikonin increases glucose uptake independent of Akt phosphorylation, via a pathway which is distinct from that activated by insulin. Conversely, in 3T3-L1 adipocytes, shikonin has been shown to increase glucose uptake by increasing Akt-phosphorylation (Kamei et al., 2002).

Fig.7: Shikonin's effect on plasma glucose in GK rats treated with DMSO/oil or shikonin for 4 days .Graph shows mean ± SEM for 6 rats.** P,0.01 for shikonin day 2 compared to day 1, ***P,0.001 for shikonin day 4 compared to day 1.

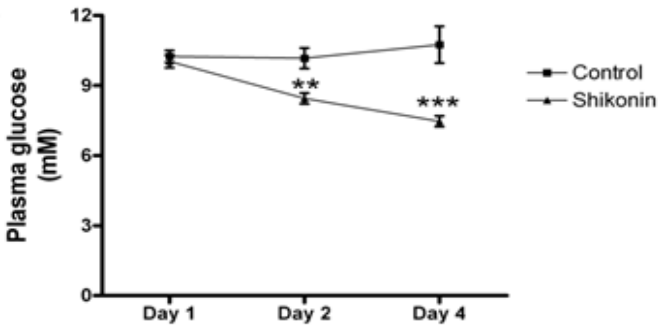
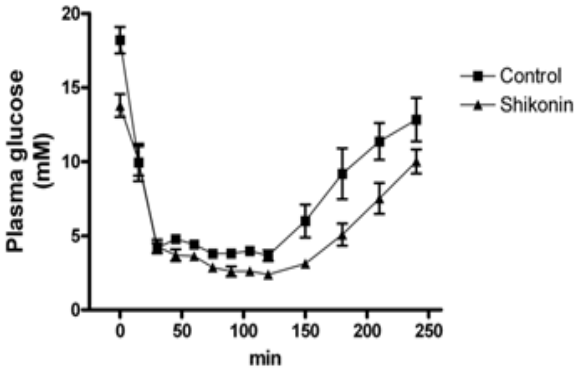


Fig.8: Shikonin's effect on insulin sensitivity in GK rats treated with DMSO/oil or shikonin for 4 days. Graph shows mean ± SEM for 6 rats.



Measurement of AMP/ATP-levels showed no differences between control- and shikonin-treated myotubes, nor was any increase in the phosphorylation of AMPK detected following shikonin treatment, indicating that shikonin does not act via AMPK. The increase in intracellular calcium levels which occurs during muscle contraction has been suggested to increase glucose uptake (Rose and Richter, 2005). Interestingly, our experiments in L6 myotubes showed that shikonin treatment does in fact lead to an increase in intracellular free calcium levels. A well-characterized molecular mechanism which leads to increased glucose uptake into skeletal muscle is the increased content and translocation of the glucose transporter GLUT4 (Koistinen et al., 2003, Chibalin et al., 2000). GLUT4-translocation has previously been suggested to be calcium-dependent, both with regards to its activation by insulin, as well as with respect to GLUT4 translocation resulting from muscle contraction (Niu et al., 2010). In attempts to identify further mechanisms in L6 myoblasts, we found that shikonin stimulated the translocation of GLUT4 from intracellular vesicles to the cell surface.

In our *in vivo* experiments in diabetic GK rats, shikonin improved glucose tolerance, as mainly reflected by lower fasting PG levels after shikonin treatment compared with placebo treatment. In an insulin tolerance test, the decrease in PG levels was similar in shikonin-treated and control rats. However, the absolute plasma glucose levels in the SCITT from 0 to 240 min were lower in shikonin-treated rats, which indicates that the effects of shikonin and insulin are additive.

In conclusion, shikonin increases glucose uptake in muscle cells via an insulin-independent pathway involving increased intracellular calcium levels and GLUT4-translocation. The beneficial effects of shikonin on glucose homeostasis demonstrate that the compound possesses properties that make it of considerable interest in the search for novel therapies for T2D.

4.3 THE NON-HEMATOPOIETIC ERYTHROPOIETIN ANALOGUE, ARA290, IMPROVES GLUCOSE TOLERANCE IN GK RATS, A MODEL OF TYPE 2 DIABETES (PAPER III)

In this paper we studied the effects of the non-hematopoietic erythropoietin analogue, ARA290, on different aspects of glucose homeostasis in spontaneously diabetic Goto-Kakizaki (GK) rats and non-diabetic control rats.

In non-diabetic Wistar rats, neither non-fasting PG levels, nor the AUCs, differed between ARA290- and placebo-treated groups during the IPGTT. In ARA290-treated GK rats, non-fasting PG levels were lower in the 3rd and 4th weeks of treatment, as compared to placebo controls ($p = 0.02$ and $p<0.05$; respectively) (Table 1). Prior to treatment, IPGTTs were similar in both groups (Fig. 9A). Moreover, during the IPGTT, the total AUC was significantly lower in the 2nd and 4th weeks in ARA290-treated, compared to control, GK rats ($p=0.003$; Fig 9B, and $p=0.02$; Fig. 9C, respectively). However, the results of the SCITTs and IPPTTs were similar in ARA290-treated and control groups, indicating that treatment with ARA290 did not influence extra-hepatic and hepatic insulin sensitivity. In addition, the hematocrit was identical in both groups. In islets isolated from ARA290-treated GK rats, the insulin responses to high glucose concentrations in relation to basal glucose levels were significantly increased, compared to islets from the placebo-treated rats ($p<0.05$). Interestingly, in response to glucose stimuli, intracellular cytosolic calcium concentrations were enhanced in islets from ARA290-treated rats, compared to islets from the placebo-treated rats ($p<0.0002$). Moreover, addition of carbachol had an effect on intracellular calcium concentrations which was additive with the significant increases resulting from ARA290-treatment, compared to the placebo-treated rats ($p<0.02$). Similar effects were seen in response to a potassium chloride stimulus ($p<0.02$).

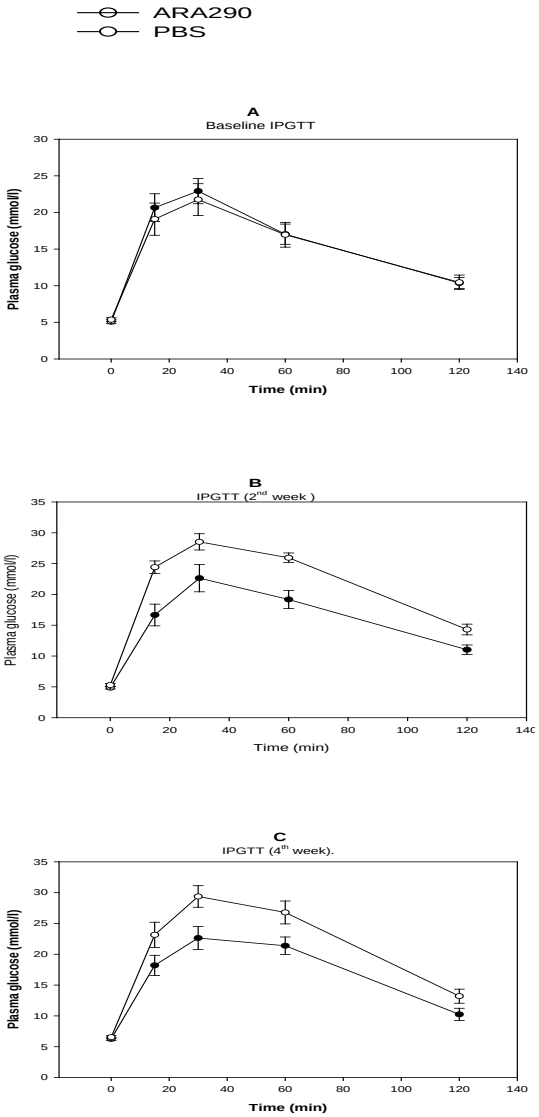
Table 1:
Plasma glucose levels (mM) in Wistar and GK rats, during 4 weeks of ARA290 (30µg/kg) and PBS treatment.Results are the mean±SEM; n, no of rats. W, week. *P<0.05 vs. control group (PBS) at the same week.

	n	W0	W1	W2	W3	W4
Wistar rats						
ARA290	6	5.6±0.2	5.9±0.1	6.3±0.2	6.3±0.2	6.3±0.2
PBS	6	5.7±0.2	5.8±0.2	6.5±0.3	6.4±0.2	6.3±0.2
GK rats						
ARA290	8	7.6±0.2	7.5±0.3	7.4±0.2	7.4±0.2*	8.0±0.2*
PBS	7	7.7±0.1	8.1±0.3	7.8±0.2	8.6±0.2	9.3±0.2

Erythropoietin (EPO) is a cytokine that regulates hematopoiesis via its binding to the erythropoietin receptor (EPO-R). EPO-R has been shown to be present in nonerythroid tissues, including pancreatic islets (Frayling, 2007). In addition to its well-known

hematopoietic action, EPO has been shown to exert anti-inflammatory, anti-apoptotic and cytoprotective effects in a wide variety of cell types (Brines and Cerami, 2006). Evidence of inflammation and apoptosis has been observed in the pancreatic islets of patients with T2D, as well as in islets from animal models of the disease (Homo-Delarche et al., 2006, Donath et al., 2003). Impaired β-cell function and insulin secretion play a primary role in T2D (Ostenson and Efendic, 2007, Frayling, 2007). Recently, EPO treatment was shown to protect against diabetes development in streptozotocin-induced and db/db mouse models of type 1 and type 2 diabetes, respectively, while exerting anti-apoptotic, anti-inflammatory, proliferative and angiogenic effects within the islets (Choi et al., 2010).

Fig 9: Plasma glucose levels during IPGTTs (A) baseline, (B) after 2 weeks and (C) after 4 weeks of treatment. Graphs show mean ± SEM.



Our present results indicate that the improvements in non-fasting PG levels as well as in glucose tolerance in ARA290-treated GK rats could be due either to an enhancement of insulin sensitivity, and/or to increased insulin secretion. Since there were no significant differences between ARA290-treated and placebo-treated controls in their responses to either the pyruvate tolerance test or the insulin sensitivity test, it is not

likely that ARA290 exerts its action on insulin sensitivity in either the liver or extra-hepatic tissues. Furthermore, as observed from the IPGTT results, fasting plasma insulin levels were not different between treated and control rats, which is in agreement with the concept that ARA290 does not improve glucose homeostasis by acting on the liver. It appears likely that ARA290–induced improvement of glucose tolerance in GK rats can be accounted for by an effect on the pancreatic β-cells, i.e. the stimulation of insulin release.

In pancreatic β-cells, intracellular calcium stores are well known to be important in regulating calcium homeostasis and to be engaged in insulin secretion (Tengholm and Gylfe, 2009). Our measurements of intracellular calcium concentrations revealed that islets from ARA290-treated rats had higher Ca^{2+} levels in response to several secretagogues, suggesting that the ARA290-induced improvement of insulin secretion could be due to an enhancement of extracellular calcium influx leading to increased cytosolic calcium concentrations.

It is known that exogenous erythropoietin increases hematocrit levels (Fenjves et al., 2003). In contrast to previous studies with EPO (Choi et al., 2010, Ogunshola et al., 2006), in which treatment enhanced hematocrit to almost 75%, the hematocrit in GK rats was entirely normal after four weeks of ARA290-treatment.

Collectively, we can conclude that treatment with ARA290 significantly improved glucose tolerance in diabetic GK rats. This effect in GK rats is rather likely due to an improvement of glucose-stimulated insulin release. The mechanism underlying this improved insulin secretion seems to be mediated by β-cell membrane depolarization and the attendant increases in intracellular calcium concentrations which participate in the signaling pathway for glucose-stimulated insulin secretion.

4.4 SEX-DIFFERENT HEPATIC GLYCOGEN CONTENT AND GLUCOSE OUTPUT IN RATS (PAPER IV)

In this paper, we characterized sex differences in carbohydrate and lipid metabolism in the rat liver. Out of approximately 3 500 gene products detected in the rat liver, 22% of the genes expressed were identified as being sex-dependent (11% being male-predominant and 11% female-predominant), as determined by whole-genome microarrays.

In line with previous reports (Soler-Argilaga and Heimberg, 1976, Soler-Argilaga et al., 1975), female-predominant genes were involved in hepatic uptake of long-chain fatty acids, synthesis of TG, and assembly of VLDL particles. Genes involved in mitochondrial and peroxisomal oxidation of FA were shown to be male-predominant. In addition, genes involved in glucose uptake and glycogen synthesis were more highly expressed in males.

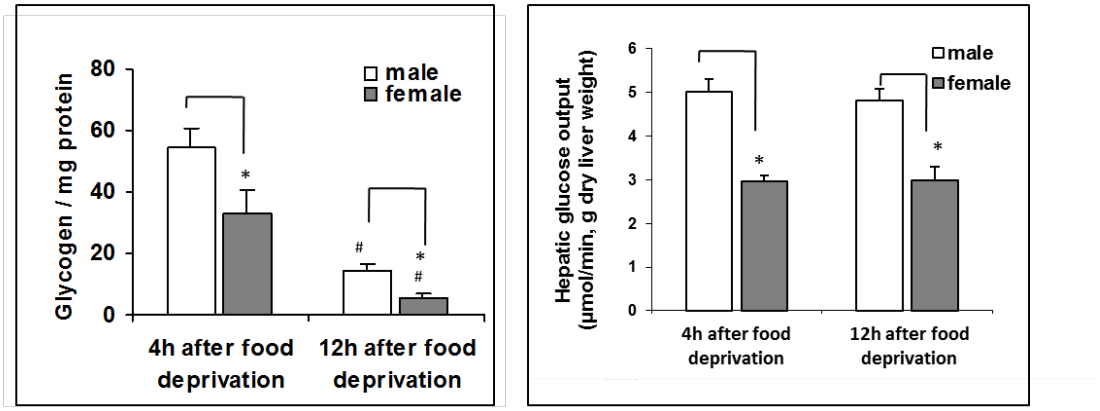
Male and female rats, fasted for either 4h or 12h, were compared with regards to differences in the levels of mRNAs encoded by genes related to metabolism in rat liver. Greater sex differences in hepatic gene expression were shown in 12 h -fasted rats, as exemplified by higher levels of acetyl-CoA carboxylase 1 (ACOX1), carnitine palmitoyltransferase 1a (CPT-1a), UDP-glucose pyrophosphorylase 2 (UGP2), glutamate oxaloacetate transaminase 1 (GOT1), glucose-6-phophatase (G6Pase),

phosphoenolpyruvate carboxykinase (PEPCK) and stearoyl-coenzyme A desaturase 1 (SCD1) in males.

After 4 h of fasting, males exhibited higher levels of hepatic glycogen content. This sex-difference was maintained even after the reduction in hepatic glycogen content seen in the 12 h -fasted rats (Fig. 10), whereas there were no sex differences in hepatic triglyceride (TG) content, FA oxidation rate, and ketone bodies in blood. Interestingly, after 4h and 12 h fasting, when perfusing the liver *in situ*, HGO was higher in perfusates collected from male rats ($P < 0.0001$) (Fig. 10), which is consistent with our gene expression studies. Males exhibited a higher ratio of insulin to glucagon at both time points. Significant decreases in PG levels were observed after 12 h of fasting in both sexes. PG levels at different durations of fasting showed no sex differences at any time point. The inconsistency between glucose levels in plasma and liver perfusates might be explained by differences in glucose uptake by peripheral tissues.

Analyses of liver perfusates, using ^{1}H-NMR spectroscopy, revealed that perfusates from male rats contain significantly higher levels of glucose ($P < 0.01$), and also significantly higher levels of lactate ($P < 0.01$), and a trend to higher levels of glycerol and the gluconeogenic amino acids glutamine and glutamate. These findings are in line with our observation of increased mRNA expression of key gluconeogenic enzymes, indicating that the male liver is more active in both producing and distributing glucose, and as well as generating metabolites important to glucose production.

Fig. 10: Effects of fasting on hepatic glycogen content and glucose output in male and female rats. Data are represented as means ± SEM, and (*) indicates a significant difference compared to the corresponding male group, whereas (#) indicates a significant difference compared to the corresponding 4h fasting group.



The reduction in glucose output after 40 min of insulin injection was greater in male rats, whereas the extent of the insulin suppressive effect on HGO was similar in male and female when expressed as a percentage of the basal glucose level. This indicates that male and female rat livers were equally sensitive to insulin, and that the sex-difference in HGO was maintained upon insulin treatment. In addition, the effects of insulin on PG levels were the same in males and females. Moreover, no sex differences were observed at the level of insulin receptor signaling with regards to Akt-phosphorylation (Ser473) 40 min after insulin treatment. AMPK was Thr172-phosphorylated to a lower degree in male livers, indicative of a higher activity through gluconeogenic pathways as compared to female livers.

In conclusion, the results of this study demonstrate higher ratios of insulin to glucagon levels, higher levels of hepatic glycogen, a lower degree of hepatic AMPK phosphorylation, higher expression levels of hepatic gluconeogenic genes and greater hepatic glucose output in healthy male rats, as compared to female rats. It is evident that fuel metabolism differs between male and female animals, as well as between men and women (Tarnopolsky and Ruby, 2001, Tarnopolsky, 2000, Devries et al., 2006).

Differences between males and females in both body composition and hepatic metabolism may lead to differences in the onset of related diseases of metabolic imbalance. Moreover, diagnoses of these diseases by metabolic profiling may be confounded by sex-specific differences in such profiles.

Also, we speculate that since males have higher rates of FA oxidation and glucose production, this may explain the increased risk for development of T2D in this sex.

5 THESIS SUMMARY

The major goal of this thesis being to investigate the effects of factors on glucose homeostasis in health and T2D, and to characterize metabolic sex-differences in the liver, the following conclusions can be made from our results:

Paper I- The study confirms that an extract of *Gynostemma pentaphyllum* (GP) exerts direct antihyperglycemic effects, as reflected by improving PG levels mainly through reducing hepatic glucose output, indicating improvements in hepatic insulin sensitivity.

Paper II- The study provides evidence that shikonin increases glucose uptake in skeletal muscle. This effect may be mediated by an insulin-independent pathway involving increased intracellular calcium levels and GLUT4-translocation in skeletal muscle.

Paper III- The study verifies that the non-hematopoietic erythropoietin analogue, ARA290, improved PG levels by increasing insulin release. Increases in intracellular calcium leading to exocytosis of insulin were detected as being involved in the potential mechanism underlying the increased insulin levels. It can be speculated that ARA290 possesses properties that make it of great interest for the development of novel treatments of T2D.

Paper IV- The study provides evidence of sex-differences at the level of hepatic carbohydrate metabolism, suggesting an increased ability in the male liver than the female liver to respond to increased energy demands.

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